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13. ABSTRACT ***(Maximum 200 Words)***

There is an urgent need for better prognostic indicators in breast cancer. We have found that: i) the p52/p46 Shc signaling protein is constitutively tyrosine-phosphorylated in most breast cancer cell lines, but not in non-tumorigenic breast epithelial cell lines; ii) expression of a 66-kDa isoform of Shc is greatly reduced in most breast cancer cell lines. The current study, then, ultimately seeks to answer the question, " Are the cellular levels of PTyr-Shc and p66 Shc of prognostic value in breast cancer? To be able to address this question, we have developed and characterized antibodies that specifically recognize, respectively, either activated Shc (phospho-specific antibodies to the Shc tyrosine317 phosphorylation site), or to the p66-Shc-specific CH2 domain. These antibodies not only will specifically immunoprecipitate their respective antigens, but will specifically detect these antigens in formalin-fixed tissue culture cells. These antibodies will be used to immunohistochemically detect and quantify the levels of these proteins in patients' tumors, and the levels will be correlated retrospectively with the aggressiveness of patients' disease.

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Raymond Frederick 7/27/00

PI - Signature Date

Table of Contents

(5) INTRODUCTION

There is an urgent need for better prognostic indicators in breast cancer. We have found that: i) the p52/p46 Shc signaling protein (which helps to link growth-factor receptors to Ras and other downstream effector pathways) is constitutively activated (tyrosine-phosphorylated) in most breast cancer cell lines (but not in non-tumorigenic breast epithelial cell lines); ii) expression of a 66-kDa isoform of Shc (that acts as a feedback down-regulator of Shc signaling to Ras) is greatly reduced in breast cancer cell lines that are dependent upon activated p52 Shc for their ability to proliferate. The current study, then, seeks to answer the question, " Are the cellular levels of PTyr-Shc and p66 Shc of prognostic value in breast cancer? Our approach to address this question is to develop and characterize antibodies that specifically recognize, respectively, either activated Shc (phospho-specific antibodies to the Shc tyrosine317 phosphorylation site), or to the p66-specific CH2 domain. These antibodies will be used to immunohistochemically detect and quantify the levels of these proteins in patients' tumors, and the levels will be correlated retrospectively with the aggressiveness of patients' disease.

(6) BODY

The body of this progress report is presented in sections according to the approved Statement of Work. The Technical Objective and Tasks for each section appear in italics. Much of this work was published in two Abstracts (attached in the Appendix) that were presented as posters at the AACR meetings earlier this year.

Technical Objective 1: Develop antibodies that specifically recognize only the tyrosine-phosphorylated, activated form of Shc and other antibodies that specifically recognize only the p66 Shc isoform

Task 1 (months 1-4): *Purify 5 mg of recombinant human p66 Shc_{1-110}.*

Inasmuch as there was a delay in receiving the human recombinant Gst-p66 Shc1-110 from our collaborator, Prof. Pelicci in Milan, Task 1 was deferred to months 8-12 for use in Tasks 4 and 5(see below), and an alternative, peptide-based strategy was used to generate rabbit antibodies to p66 Shc (see Task 3, below).

Task 2 (months 1-5): *Finish our ongoing characterization of Rabbit #58 antibodies specific for the active state (PY317) of Shc.*

In the original grant proposal we had shown preliminary evidence that rabbit #58 produced antibodies that by immunoprecipitation appeared rather specific for the PY317 active state of Shc (see Fig. 5 in the original proposal). During the first period of this grant, we further assessed this antibody's fine specificity, first by Western blotting. For this purpose, we

made use of the HBL-100 breast epithelial cell lines that we had engineered to express either a wild type GST-human recombinant p52 Shc fusion protein (clone 26), a dominant negative GST-human recombinant p52 Shc fusion protein in which the 317 tyrosine had been mutated to a phenylalanine (clone 17), or an empty vector control (clone 9). As can be seen in Figure 1(Appendix), the expression of either the wt-Shc or the dn-Shc nearly completely blocked the ability of EGF to induce tyrosine phosphorylation of the endogenous p46, p52, and p66 Shc proteins. Instead in response to EGF, the wild type GST-Shc is heavily tyrosine phosphorylated (on residues 239 and 317) and the dn-Shc is lightly tyrosine phosphorylated (only on residue 239...since its 317 tyrosine residue has been mutated to a phenylalanine), and both of these expressed recombinant Shc proteins are tightly associated with EGF-induced tyrosine phosphorylated EGF receptor. These cells, then, are excellent prototypes to examine the fine specificity of the rabbit #58 antibody by immunoprecipitation and by immunocytochemistry. For, if the rabbit#58 antibody is indeed specific for PY317 Shc, we would it expect it to immunoprecipitate only the tyrosine phosphorylated wt-GST-Shc from EGF-stimulated clone 26 cells. Indeed, this was the case (Fig. 2, Appendix). The rabbit 58 antibody precipitated the tyrosine(239,317) phosphorylated wt-GST-Shc protein from EGF-stimulated clone 26 cells, but did not precipitate the tyrosine (239)-phosphorylated dn-GST-ShcY317F protein from clone 17 cells. Various controls showed that the rabbit #58 binding could be completely inhibited by the immunizing peptide, and that the antibody did not bind to other proteins that had been tyrosine phosphorylated in response to EGF. Importantly, the rabbit #58 antibodies showed similar specifity for PY317 Shc by immunocytochemical staining of EGF-stimulated clone 26 and clone 17 cells (Fig. 2, lower panels, Appendix). An interesting comparison of the rabbit 58 anti-PY-Shc immunocytochemical staining intensity and patterns in EGF-stimulated parental HBL-100 cells with the staining patterns for total Shc proteins (RαShc), and total phosphotyrosine (MαPtyr) can be seen in Fig. 3 (Appendix). In particular, notice that most of the tyrosine-phosphorylated Shc protein is located in perinuclear regions, presumably in the trans-golgi and/or lysosomes.

We next needed to determine if the rabbit 58 antibody to PY-Shc would positively stain breast cancer cell lines that we knew to contain PY-Shc from our earlier immunoblotting experiments (Fig. 2C in the original grant proposal; published in Stevenson et al., 1998, and partially reproduced here in Fig. 4A, Appendix, for the readers benefit). Notice that all of the cell lines that contained significant levels of PY-Shc by immunoblotting (Fig. 4A, Appendix), also exhibited strong immunocytochemical staining

with rabbit 58 antibody (Fig. 4B, Appendix). In contrast, a non-transformed breast epithelial cell line, Hs-578Bst, and the single breast cancer cell line, MDA-MB-231, that lack significant levels of tyrosine phosphorylated Shc by immunoblotting (Fig. 4A), also fail to stain with the PY-Shc specific rabbit 58 antibody. The only caveat with the immunocytochemical staining is the rather high amount of non-specific background, due not to any non-specificity with our rabbit 58 antibody, but rather an inherent feature of the DAKO CSA staining system. This non-specific staining is reportedly due to endogenous biotin-containing molecules (DAKO, presonal communication). DAKO has developed an improved CSA system that avoids this problem, and has offerred to have us beta-test it with the PY-Shc-specific antibody.

Task 3 (months 4-8): *Immunize, boost, bleed, affinity purify antibodies [reactive with p66 Shc] from two NZW rabbits.*

Our original plan called for developing antibodies specific for p66-Shc, by immunizing rabbits with human recombinant Gst-p66 Shc_{1-110}. However, because of the delay in receiving the human recombinant Gst-p66 Shc1-110 from our collaborator, we chose to use our alternative strategy, which was to immunize rabbits with a hydrophilic peptide derived from p66 Shc_{1-110} region. Accordingly, the peptide, SerGlySerThrProProGluGluLeuProSerProSerAlaSerSerLeu, was coupled to the carrier protein, KLH, and used to immunize three New Zealand White rabbits, employing a standard, 10-week immunization/challenge protocol. The antisera collected at 10 weeks were tested for reactivity against the immunizing peptide in a solid phase ELISA. Antisera from one of the three rabbits, rabbit #405, reacted with the immunizing peptide (titer of about 1/5000). The peptide-specific antibodies were affinity purified from rabbit #405 antisera on a Sepharose matrix to which peptide had been covalently linked. The fine specificity of these antibodies was then characterized in Task 4, below.

Task 4 (months 6-18): *Characterize the antigenic fine specificity of the purified antibodies [specific for p66 Shc].*

p66 Shc-specific antibody that had been affinity purified from rabbit #405 antisera (see Task 3, above), was tested for its ability to recognize human recombinant GST-p66 Shc CH2 domain (a fusion protein comprised of GST and the first 110 amino acids of p66 Shc...the amino acids unique to p66 Shc compared to the p52 Shc isoform). The human recombinant Gst-p66 Shc_{1-110} bacterial expression vector was obtained from Prof. Pelicci, in Milan, Italy. We expressed this protein in E. coli, and affinity purified it using a glutathione affinity matrix. SDS PAGE analysis and Coomassie

blue staining revealed that the purified protein consisted of three major bands near the predicted molecular weight of the fusion protein, along with a faster migrating band, and a much slower migrating group of minor bands. Immunoblotting with antibodies to GST revealed that all of the bands contained GST (Fig. 5, Appendix). The rabbit #405 antibodies reacted with all but the fastest migrating species(Fig. 5). We interpret this to mean that the fastest moving band contains only GST (or GST and a small piece of p66CH2 that does not contain the p66 epitope used for immunization). The other bands near the predicted molecular size likely are the intact GST-p66 Shc CH2 fusion protein and proteolytic clips of this protein, while the highest molecular weight proteins are almost certainly dimers of GST-p66Shc CH2 and its proteolytic fragments caused by GST mixed disulfide reactions (unpublished observations). Thus, not only does the antisera raised against the small CH2 peptide recognize the whole CH2 domain, but it can do this on immunoblots and appears relatively specific (it did not react with the GST-only band).

We sought to further test the specificity of the rabbit #405 antibody by asking if it would specifically immunoprecipitate p66 Shc. To test this, we took advantage of our prior findings (see Fig. 4A), that the breast epithelial cell line, HBL-100, contains normal levels of p66 Shc, comparable to levels of p52 Shc, while the breast cancer cell line, MDA-MB-453, lacks detectable p66 Shc. The rabbit #405 antibody cleanly and clearly precipitated only the p66 Shc protein (Fig. 6).

Immunocytochemical staining of the HBL-100 and MDA-MB-453 cells revealed that the rabbit #405 antibody also could specifically detect p66 Shc in the HBL-100 cells (Fig. 6). However, the non-specific background staining noted in Task 3 was more problematic here. We anticipate that the re-designed DAKO CSA kit will eliminate or greatly reduce this problem.

Task 5 (months 16-26): *Develop and characterize monoclonal antibodies specific to PY-Shc peptide*

Although the actual monoclonal antibody development is not slated to begin until year two of this grant, there are well-known immunologic advantages to be gained by allowing a long immunologic rest period in between immunization and the final challgenge just before hybridoma development. Therefore, we recently immunized 10 BALB/c mice with the the PY[317]-Shc peptide-KLH conjugate (N-acetyl-LeuPheAspAspProSer([P]Tyr)ValAsnValGlnAsnLeuCys)(corresponding to human Shc amino acids 311 to 323, with an added cysteine C-terminus to facilitate maleimide coupling to the carrier protein, Keyhole Limpet haemocyanin). ELISA testing of an early bleed indicates that three of

these mice are making antibodies specific to the PY-peptide. These three mice will be challenged further with the peptide antigen, and their antisera will be characterized for PY-Shc specificity in a manner similar to that shown above for the polyclonal rabbit antisera. The mouse with the highest titer of specific antibody will be used to develop the anti-PY[317]-Shc hybridomas.

Task 6 (months 26-36): *Develop and characterize monoclonal antibodies specific to p66 Shc isoform*

We have expressed, purified and quality controlled the Gst-p66 Shc CH2 fusion protein that we plan to use later to develop monoclonal antibodies specific to p66 Shc. See progress reported under Task 4 and Fig. 5.

Technical Objective 2: Use these antibodies to determine the cellular levels of constitutive p52 Shc tyrosine phosphorylation and to determine the cellular levels of the p66 Shc isoform in panels of breast-cancer specimens; correlate the level of activated p52 Shc and the level of p66 Shc with the presence of other markers (such as estrogen receptor, ErbB2, EGF receptor, hormone responsiveness), and with clinical disease and pathology (presence or absence of lymph-node metastases, disease-free survival, overall survival).

Task 6 (months 6-36): *Test freshly isolated breast-cancer cells for levels of Shc tyrosine phosphorylation, p66 Shc, ErbB2, EGF receptor and estrogen receptor. Correlate with the presence of lymph-node metastases. This will be necessary only in the unlikely case that the antibodies did not recognize activated Shc or p66 Shc in the formalin-fixed specimens.*

The rabbit antibodies to PY[317]-Shc and to p66 Shc function well in formalin-fixed specimens(see the reports for Tasks 2-4), so this contingent Task will not be required.

Task 7 (months 11-30): *Immunostain for PY-Shc and p66 Shc sections from 300 archived paraffin blocks (Roger Williams Hospital Pathology Dept) containing breast-cancer specimens for level of activated Shc, ErbB2, EGF receptor and estrogen receptor. Immunostain sections from blocks pre-screened by David Stern for levels of activated ErbB2. Immunostain sections from blocks pre-screened by Edward Filardo for active MAP kinase. Correlate with estrogen receptor status, the presence of lymph-node metastases, disease-free survival and overall survival.*

We have temporarily deferred beginning these studies while we await and test the new, lower-background CSA staining kit from DAKO.

Task 8 (months 20-36): *Statistical analyses of the data and preparation for publication.*

MATERIALS AND METHODS:

ANTISERA

PY[317]-Shc: New Zealand white rabbits (#58 and #73) were immunized with an N-acetylated tyrosine-phosphorylated Shc peptide:
N-acetyl-LeuPheAspAspProSer([P]Tyr)ValAsnValGlnAsnLeuCys (corresponding to human Shc amino acids 311 to 323, with an added cysteine C-terminus to facilitate maleimide coupling to the carrier protein, Keyhole Limpet haemocyanin). Sera was collected 10 weeks later after two booster immunizations.

p66Shc-CH2: Three New Zealand white rabbits were immunized with a p66 peptide: **SerGlySerThrProProGluGluLeuProSerProSerAlaSerSerLeu** (corresponding to a hydrophilic region within the human Shc amino acids 1 to 110 in the unique CH2 domain of the p66Shc isoform). Sera was collected 10 weeks later after two booster immunizations. Only one rabbit of the three demonstrated titratable antibodies to $p66^{Shc}$, as determined by ELISA monitoring.

Other antibodies: RαShc antibody, which recognizes all phosphorylated or unphosphorylated isoforms of Shc, and the 4G10 monoclonal antibody, which recognizes phosphotyrosine, were purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

CELLS

HBL-100 cells (American Type Culture Collection [ATCC], Rockville, MD) are a non-tumorigenic human breast-epithelial cell line that express a high level of EGF receptors. HBL-100/wt Shc 26 and HBL-100/dn Shc 17 were derived by G418 selective pressure after Lipofectamine-Plus transfection with pEBG vector carrying the cDNA (with expression driven by the constitutive EF1α promoter) for either wild-type $p52^{Shc}$-GST or a dominant-negative mutant $p52^{Shc}$Y317F-GST, respectively. SKBR3 cells (ATCC) and MDA-MB-453 cells (ATCC) are transformed epithelial cell lines derived from human breast cancer that overexpress the ErbB2 member of the EGF-receptor family and therefore, have constitutively tyrosine-phosphorylated Shc. SKBR3 express all three Shc isoforms; MDA-MB-453 do not express the $p66^{Shc}$ isoform.

ELISA FOR ANTIBODIES

Immunlon II microtiter wells were coated with PY-Shc or $p66^{Shc}$-CH2 peptide (1 μg/50 μl) in PBS, blocked with 1% BSA in PBS, and then incubated sequentially with the respective test rabbit antibodies, with a conjugate of horse-radish peroxidase linked to donkey antibody to rabbit immunoglobulin, and finally with the chromogenic HRP substrate, o-phenylenediamine. ABS 450 nm was monitored.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Cells were either stimulated or not stimulated with EGF (100 ng/ml for 10 or 20 min), as indicated, and then extracted with 1% Triton X-100 in a buffer containing protease, kinase and phosphatase inhibitors. Proteins were immunoprecipitated with either the new antibodies or commercial antibodies to Shc or PY. These antibodies were pre-bound to protein A-sepharose beads and stabilized by crosslinking with dimethylpimelimidate (DMP). The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the specified antibodies. Bound antibodies were then detected by enhanced chemiluminescence (ECL).

For some experiments, the cells were serum starved in phenol red-free medium prior to growth factor stimulation and extraction.

IMMUNOCYTOCHEMISTRY

Cells were seeded into chamber slides and incubated until reaching approx. 60-65% confluence. Cells were serum starved in phenol red-free medium before some chambers were stimulated with EGF (100 ng/ml for 20 min). Cells were rinsed with PBS and then fixed in 10% buffered-formalin for 10 min. Immunocytochemical staining was carried out according to the manufacturer using either the Catalyzed Signal Amplification (CSA) Peroxidase System (DAKO, Carpinteria, CA) following Target Retrieval with the CSA Ancilliary System (DAKO). Primary antibodies: MαPtyr (5 μg/ml); RαShc (5 μg/ml); αR58-Pre-Immune (70 μg/ml or 7 μg/ml); αR58 (10 μg/ml or 1 μg/ml); αp66-Pre-Immune (80 μg/ml or 8 μg/ml); and αp66 (40 μg/ml or 4 μg/ml) were diluted in Background Reducing Components (CSA Ancillary System) and incubated with respective cell samples for 1 h at room temperature. Primary antibody was detected by incubating for 15-30 min with one of the following: biotinylated rabbit anti-mouse or goat anti-rabbit, or secondary antibody conjugated to HRP. In some cases, the antigen-antibody signal was then amplified through several steps before detection by DAB precipitation at the antigen site.

(7) KEY RESEARCH ACCOMPLISHMENTS:

- Development and characterization of polyclonal rabbit antibody that specifically recognizes Shc only when it is phosphorylated on tyrosine 317. The antibody functions in immunoprecipitations and immunocytochemistry of formalin-fixed specimens. It accurately detects tyrosine phosphorylated Shc in several breast cancer cell lines.

- Development and characterization of polyclonal rabbit antibody that specifically recognizes only the p66 Shc isoform. This antibody also functions in immunoprecipitations and immunocytochemistry of formalin-fixed specimens.

(8) REPORTABLE OUTCOMES:

Two abstracts and poster presentations were given at the AACR meetings in March:

Davol, P.A. and Frackelton, A.R.Jr. Development and characterization of state-specific antibodies recognizing only activated, tyrosine-phosphorylated Shc. Proc. Amer. Assoc. Can. Research 41:97-98, 2000.

Davol, P.A. and Frackelton, A.R.Jr. *In Situ* detection of tyrosine-phosphorylated Shc protein as an indicator of growth factor-receptor

activation in breast cancer cells. Proc. Amer. Assoc. Can. Research 41:336, 2000.

(9) CONCLUSIONS:

We have developed polyclonal rabbit antibodies that can accurately detect the presence of elevated levels of tyrosine phosphorylated Shc, and other antibodies that can detect the presence of the p66 Shc isoform in formalin-fixed tissue culture specimens. Non-specific background staining due to endogenous biotin will soon be reduced with the availability of an improved kit design from DAKO. With this improvement, we will be able to use these antibodies with archived breast cancer specimens, and will be able to determine if the tumor levels of PY-Shc and p66 Shc are of prognostic value.

(9) REFERENCES CITED:

Stevenson LA, and Frackelton AR Jr. Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: Correlation with ErbB2 and p66 Shc expression. Breast Cancer Res & Treat, 49:119-128, 1998.

APPENDIX

ABSTRACTS (2)

FIGURES (1-6)

Proc. AACR 41:97-98, 2000

#622 DEVELOPMENT AND CHARACTERIZATION OF STATE-SPECIFIC ANTIBODIES RECOGNIZING ONLY ACTIVATED, TYROSINE-PHOSPHORYLATED SHC. Pamela A Davol, and A R Frackelton, Jr., *Brown Univ, Providence, RI, and Roger Williams Med Ctr, Providence, RI*

Activation of growth factor-receptor tyrosine kinases is implicated in neoplastic progression and poor prognosis. However, because receptor types and activities vary from tumor to tumor, we hypothesized that a common downstream-signaling component of such receptors would indicate an integrated read-out of total receptor activity and thus, provide a better indicator for patient prognosis. To this end, we have developed rabbit antibodies that specifically recognize only activated, tyrosine phosphorylated (PY) Shc: an adapter-protein, tyrosine-phosphorylated by activated receptors, that propagates the signaling cascade. Antibodies were purified by immunospecific affinity chromatography on the Shc phosphopeptide covalently linked to a solid matrix Antibodies from two rabbits, #58 and #73, demonstrated high titer by ELISA assay against immunizing peptide and clear specificity for Shc by immunoprecipitation (ip). However, ip of Shc protein by R73 antibodies (αR73) from the non-transformed breast epithelial cell line, HBL-100, in the absence of epidermal growth factor (EGF) stimulation suggests that (αR73 immunoprecipitates Shc regardless of whether Shc is PY. In contrast, R58 antibodies (αR58) recognize only the activated PY form of Shc as indicated by the ability of αR58 to detect Shc protein only in HBL-100 cells stimulated with EGF, as well as constitutively activated Shc in the SKBR3 breast cancer cell line. Furthermore, αR58 does not ip other PY proteins such as ErbB2 suggesting that αR58's specificity is not directed to PY alone. These results suggest the feasibility of generating antibodies specific for PY forms of Shc. αR58 will provide the means by which the level of activated Shc is assessed as a prognostic indicator in a retrospective study of a variety of clinical breast cancer specimens. [Supported by Dept. of Defense Breast Cancer Grant BC980415]

Proc. AACR 41:336, 2000

#2132 *IN SITU* DETECTION OF TYROSINE-PHOSPHORYLATED SHC PROTEIN AS AN INDICATOR OF GROWTH FACTOR RECEPTOR-ACTIVATION IN BREAST CANCER CELLS. Pamela A Davol, and A R Frackelton, Jr., *Brown Univ, Providence, RI, and Roger Williams Med Ctr, Providence, RI*

Considerable evidence suggests that poor prognosis in breast cancer correlates with increased activation of certain growth factor receptor tyrosine kinases. A signaling protein in common with all of these kinases is the adapter protein, Shc, which is phosphorylated in response to receptor activation. To evaluate activated-Shc as a prognostic indicator, we have developed an antibody, αR58, which specifically recognizes only tyrosine-phosphorylated Shc (PY-Shc). Immunocytochemical (ICC)-specificity of αR58 was confirmed in formalin-fixed, non-transformed breast epithelial cells with or without prior receptor stimulation. HBL-100s were transfected to overexpress wild-type p52 Shc (wt-Shc), which is preferentially phosphorylated over endogenous Shc during receptor activation, or mutant p52 Shc (Y317F-Shc), which lacks the antibody-specific tyrosine[317]-phosphorylation site. Unstimulated wt-Shc and Y317F-Shc cells, or EGF-stimulated Y317F-Shc cells demonstrated only background staining for αR58 consistent with steady-state receptor inactivation in normal breast epithelial cells and absence of Shc tyrosine[317]-phosphorylation in mutant-transfectants. In contrast, EGF-stimulation of wt-Shc cells produced a dramatic increase in αR58-immunolabeling consistent with growth factor-induced PY-Shc. Importantly, in an evaluation of normal and malignant breast cell lines, αR58 detected high (MDA-MB-453, MDA-MB-361, SKBR-3, BT-474), moderate (MCF-7, T47D, ZR-75-1) or background (MDA-MB231, HBL-100, Hs578Bst) levels of constitutive PY-Shc that paralleled Shc-immunoprecipitated/PY-immunoblots. αR58-specificity for PY-Shc by ICC analysis is the foundation of current studies evaluating PY-Shc in panels of breast cancer specimens as a prognostic indicator for clinical disease. [Supported by Dept. of Defense Breast Cancer Grant BC980415]

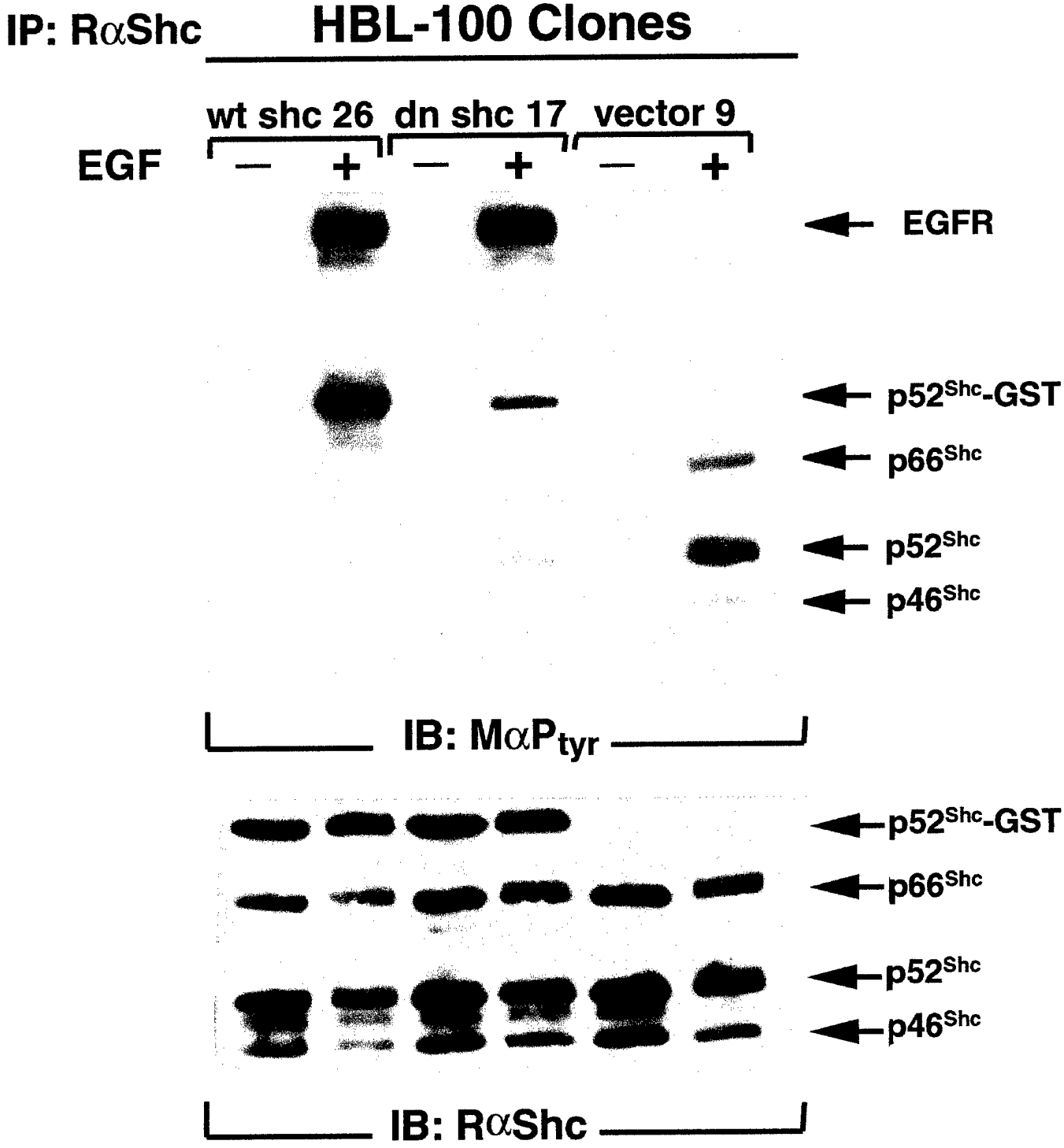


Figure 1. HBL-100 clones transfected to constitutively express a wild-type $p52^{Shc}$-GST fusion protein (wt shc 26) or a dominant-negative Tyr_{317}-mutant $p52^{Shc}$-GST fusion protein (dn shc 17) demonstrate an increase in phosphorylation of tyrosine residues (317 and/or 239/240) on the fusion protein compared to endogenous Shc proteins.

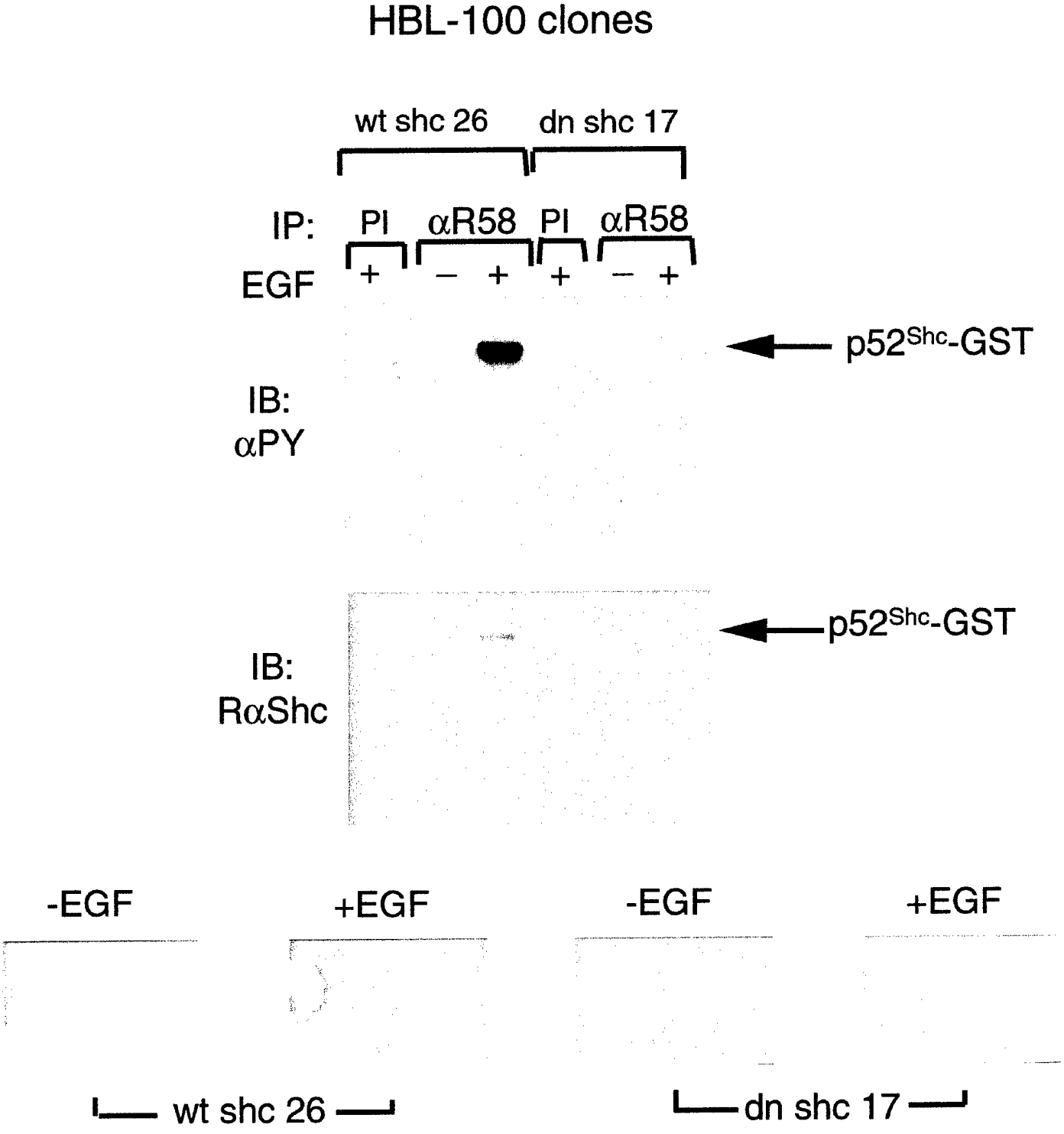


Figure 2. αR58 antibodies are specific for the phosphorylated tyrosine$_{317}$ residue of the Shc protein. Absence of Shc detection in EGF-non-stimulated wt shc 26 clones indicates that αR58 recognizes only the activated, tyrosine phosphorylated form of Shc. Furthermore, the inability of αR58 to detect Shc in EGF-stimulated dn shc 17 clones, which lack only the tyrosine$_{317}$ phosphorylation site, indicates that αR58 specificity is not directed to phosphotyrosine alone. These findings are consistent for both immunoprecipitation and immunocytochemical detection.

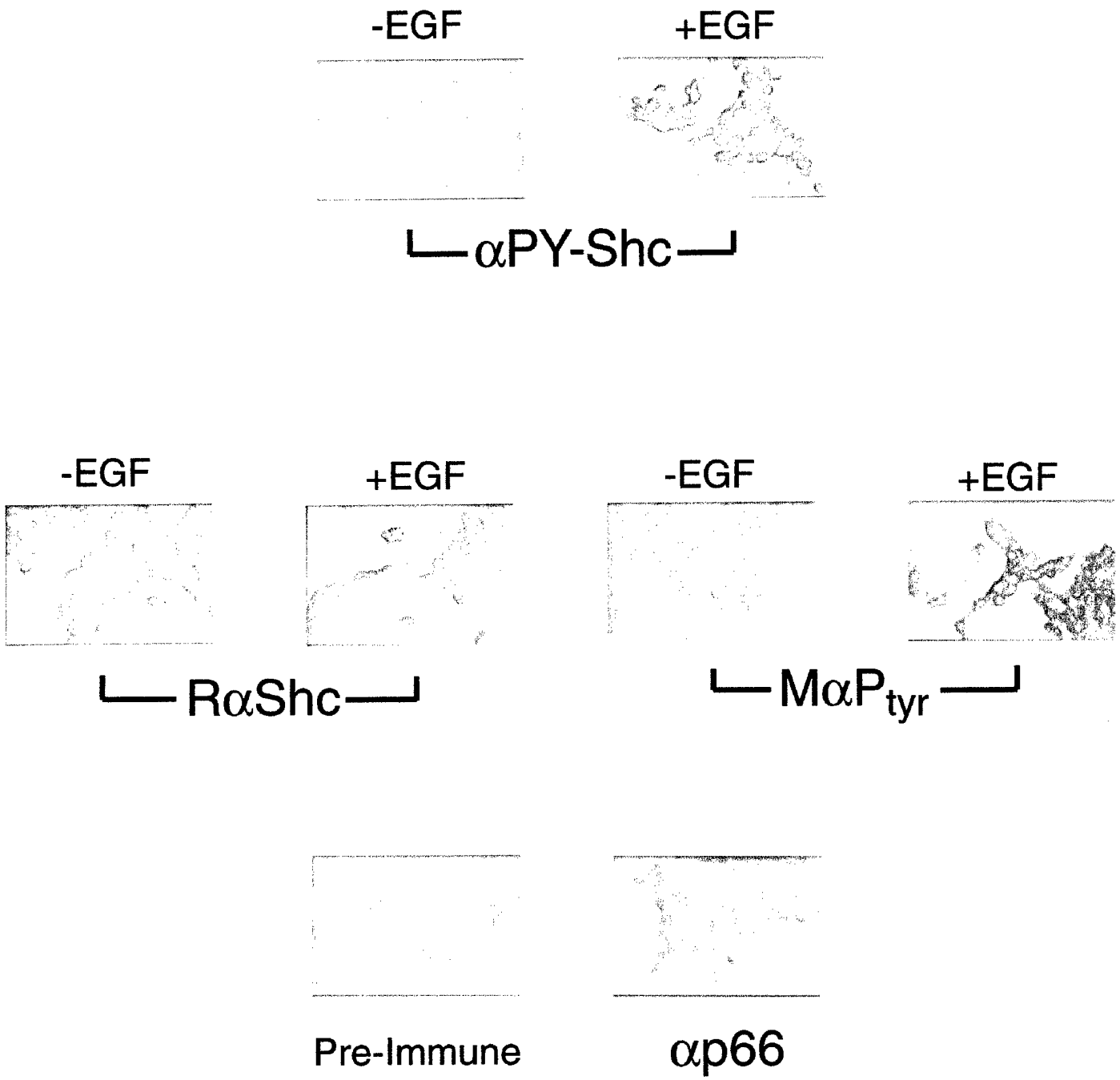
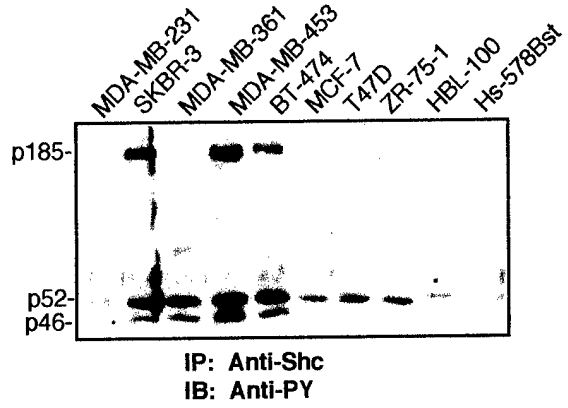


Figure 3. Antigenic specificity of antibodies by immunocytochemical detection in formalin-fixed HBL-100 cells.



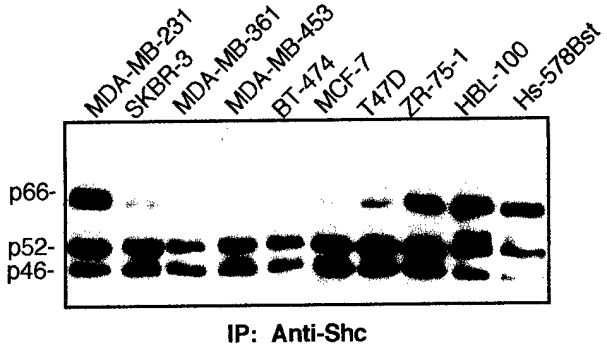


Figure 4(A). Constitutively tyrosine-phosphorylated Shc (PY-Shc) in breast cancer cells. Upper Panel: Detergent extracts of breast cell lines were immuno-precipitated with Shc antibody and blotted with antibody to PY. Only HBL100 and Hs-578Bst are non-transformed cell lines.
Lower Panel: Reprobe of blot in Panel A with antibody to Sic.

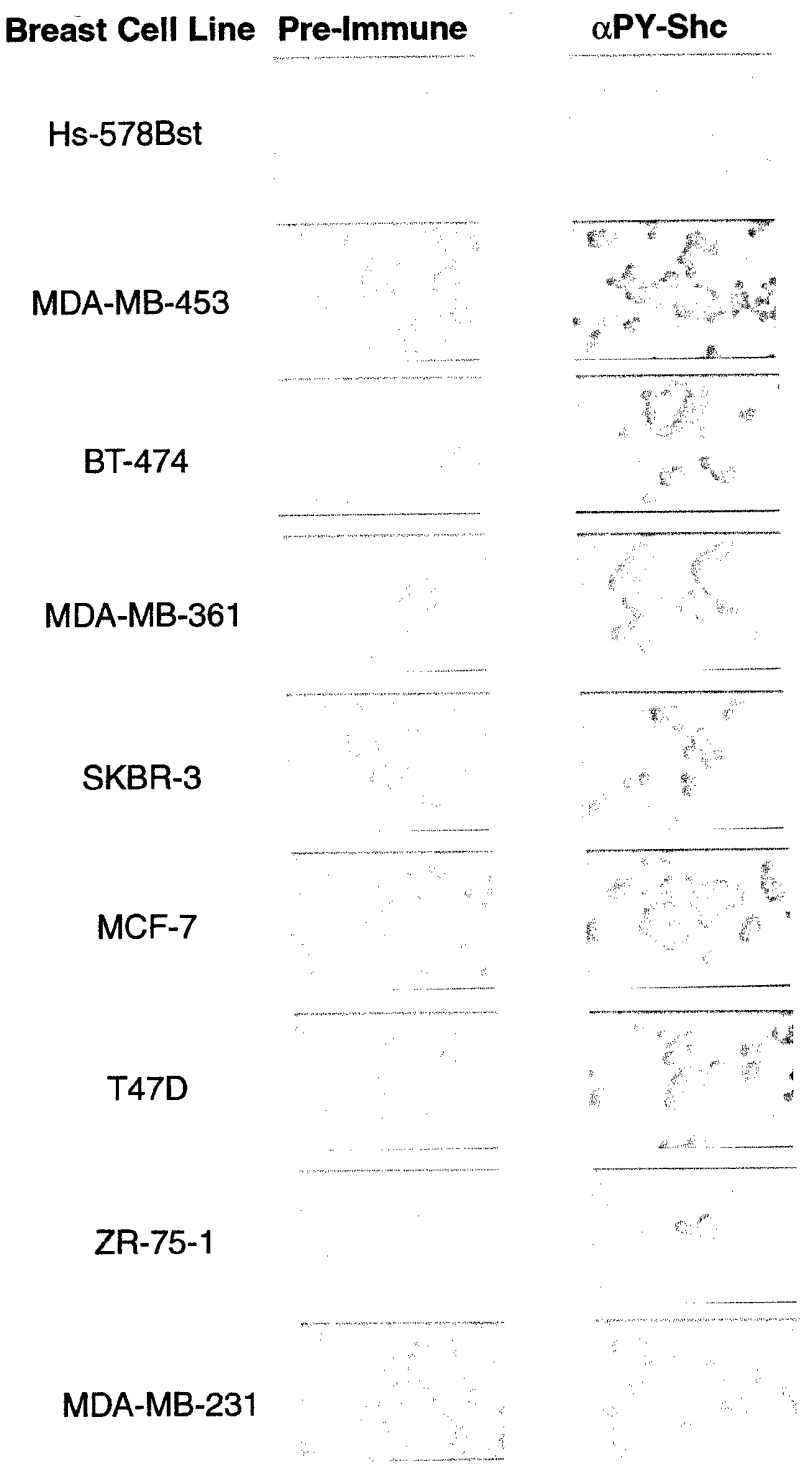
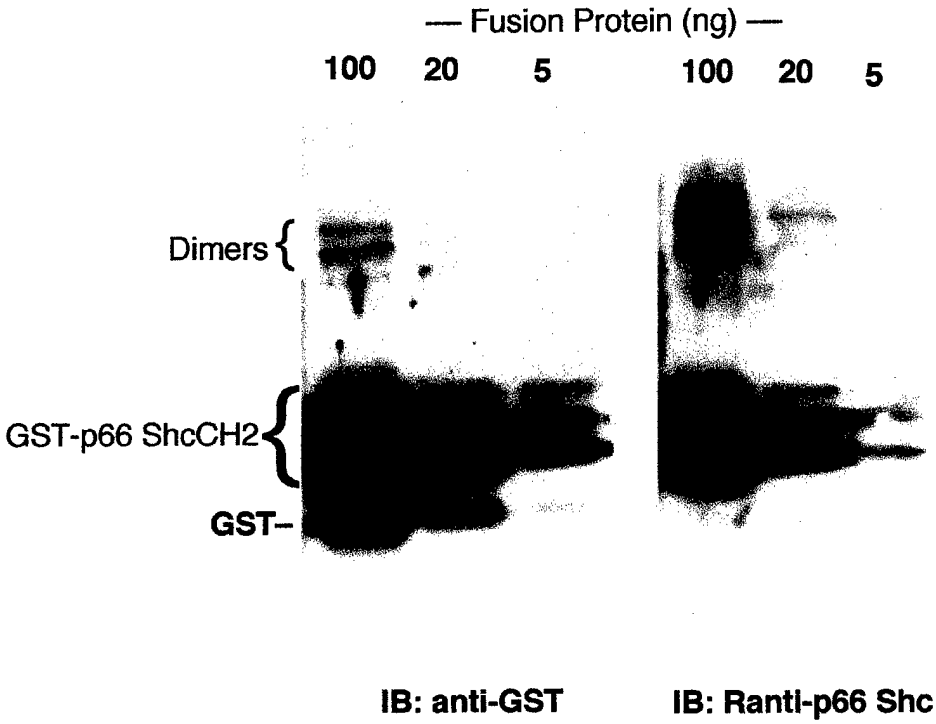
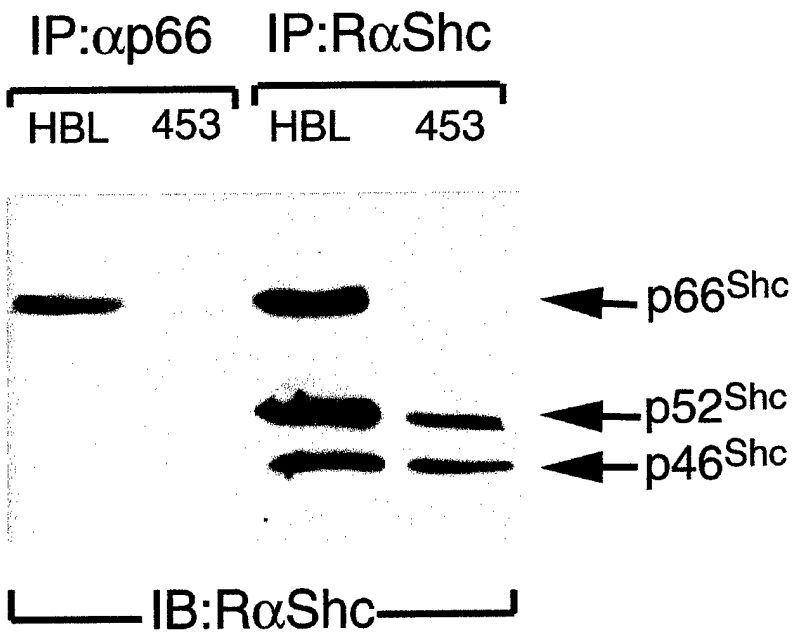


Figure 4(B). Immunocytochemical detection of tyrosine[317]-phosphorylated Shc in formalin-fixed breast cancer cell lines.

Figure 5. Analysis of the Recombinant Human GST-p66 ShcCH2 fusion protein by anti-GST and anti-p66Shc peptide immunoblotting





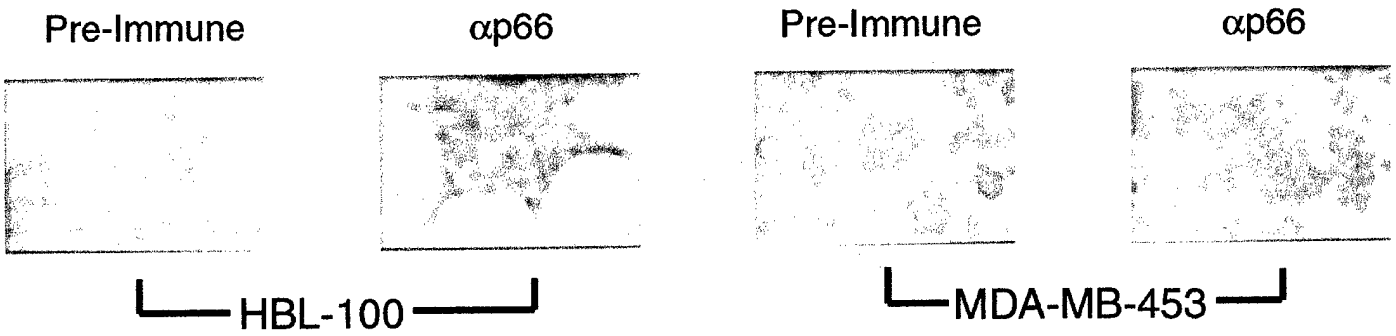


Figure 6. Rabbit antibodies raised to a peptide from the p66 Shc CH2 domain specifically recognize the p66 Shc isoform by immunoprecipitation (upper panel) and by immunocytochemical staining of formalin-fixed tissue culture cells.